Costimulation through TLR2 drives polyfunctional CD8$^+$ T cell responses

Short title: TLR-mediated costimulation promotes cytokine production

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Abstract

Optimal T cell activation requires antigen recognition through the T cell receptor (TCR), engagement of costimulatory molecules, and cytokines. T cells can also directly recognize danger signals through the expression of toll-like receptors (TLRs). Whether TLR ligands have the capacity to provide costimulatory signals and enhance antigen-driven T cell activation is not well understood. Here, we show that TLR2 and TLR7 ligands potently lower the antigen threshold for cytokine production in T cells. To investigate how TLR triggering supports cytokine production, we adapted the protocol for flow cytometry-based fluorescence *in situ* hybridization (Flow-FISH) to mouse T cells. The simultaneous detection of cytokine mRNA and protein with single-cell resolution revealed that TLR triggering primarily drives *de novo* mRNA transcription. *Ifng* mRNA stabilization only occurs when the TCR is engaged. TLR2, but not TLR7-mediated costimulation, can enhance mRNA stability at low antigen levels. Importantly, TLR2 costimulation increases the percentage of polyfunctional T cells, a hallmark of potent T cell responses. In conclusion, TLR-mediated costimulation effectively potentiates T cell effector function to suboptimal antigen levels.

36 **Introduction**

37 $CD8^+$ T cell responses are critical to defend our body from insults. They promote the
38 clearance of primary infections, protect us from previously encountered pathogens, and
39 can control and kill tumor cells. To exert their effector function, T cells produce high
40 levels of effector molecules, such as the key pro-inflammatory cytokines interferon-γ
41 (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2). T cells that produce
42 two or more cytokines, also known as 'polyfunctional' T cells, identify the most potent
43 effector T cells against infections and malignant cells (1-5). The induction of
44 polyfunctional T cell responses thus remains the ultimate goal of vaccine and T cell
45 therapy.

46

47 T cell effector function as defined by the magnitude of cytokine production depends on
48 three signals: 1) triggering of the T cell receptor (TCR), 2) engagement of costimulatory
49 molecules, and 3) availability of pro-inflammatory cytokines (6-8). All three signals are
50 required for optimal priming of naive T cells (9, 10). In addition, the coordinated
51 engagement of two or more signals can potentiate the activity of effector and memory T
52 cells (11-13). The intensity of TCR signaling can vary depending on the amount and
53 affinity of the antigen (14-16) and, upon suboptimal TCR engagement, triggering of
54 costimulatory molecules, such as CD28, significantly decreases the threshold of T cell
55 activation (17-21). In addition, cytokines like type I IFNs, IL-12 and IL-18 can promote
56 IFN-γ production both on their own, and in combination with TCR engagement (22-25).

57

58 During infection, T cells are also exposed to danger signals, which can be sensed by
59 pattern recognition receptors, such as Toll-like receptors (TLRs). RNA-seq analysis
60 revealed that long-lived human memory T cells express a strong TLR-signature, which is
61 absent in naive T cells (26). In addition, TLR2 expression on human T cells was
62 significantly increased upon TCR activation (26, 27), and TLR2 engagement was
63 suggested to reduce the minimal TCR threshold required for T cell proliferation and
64 memory formation (28). The acquired expression of TLRs on T cells suggests that TLRs
65 may support the effector function of differentiated T cells. Indeed, we and others showed
66 that effector and memory $CD8^+$ T cells - but not naive T cells - can directly respond to
67 TLR2 and TLR7 ligands by producing IFN-γ in an antigen-independent manner (29, 30).
68
69 At the molecular level, TLR-triggering alone on T cells specifically drives *de novo*
70 transcription of short-lived *Ifng* mRNA, which promotes a short induction of cytokine
71 production (29). Conversely, the engagement of the TCR also engages post-
72 transcriptional mechanisms that increase the stability of cytokine mRNA and/or drives
73 their translation (29, 31). These post-transcriptional regulatory events are required to
74 reach optimal magnitude and kinetics of cytokine production (31), and can be potentiated
75 by costimulatory signals, such as engagement of CD28 or LFA-1 (32, 33). Whether TLR
76 ligands can also augment cytokine production of TCR triggered effector $CD8^+$ T cells
77 and, if so, which mechanisms they employ is yet to be determined.
78
79 Here, we show that TLR2 and TLR7 ligands lower the antigen threshold required for
80 cytokine production of effector $CD8^+$ T cells, yet by employing different mechanisms of

81 regulation. Whereas costimulation through TLR7 only induces *de novo* mRNA
82 transcription, TLR2 signaling synergizes with the TCR to also prolong the half-life of
83 *Ifng* mRNA. The engagement of mRNA stabilization supported by TLR2 ligands
84 correlates with enhanced polyfunctional capacity of T cells. Thus, our data demonstrate
85 that distinct stimuli can differently integrate with the TCR signaling to fine-tune T cell
86 responses. Unravelling the direct contribution of TLR triggering to T cell effector
87 functions might be exploited in the future to rationalize the use of TLR ligands as
88 adjuvants for vaccination strategies and T cell therapies.

Materials and Methods

Mice, human PBMCs and cell culture

C57BL/6J mice and C57BL/6J.OT-I TCR transgenic mice (OT-I) were housed and bred in the animal department of the Netherlands Cancer Institute (NKI). Experiments were approved by the Experimental Animal Committee (DEC) and performed in accordance with institutional, national and European guidelines.

Studies with human T cells were performed in accordance with the Declaration of Helsinki (Seventh Revision, 2013), and buffy coats were obtained from healthy donors with written informed consent (Sanquin).

Cells were cultured in IMDM (GIBCO-BRL) supplemented with 8% FCS, 2mM L-Glutamine, 20U/mL penicillin G sodium salts, and 20µg/mL streptomycin sulfate. For mouse T cells, MEC.B7.SigOVA cells, and B16F10 melanoma cells or B16F10 melanoma cells expressing the C-terminal part of ovalbumin (B16-OVA; (34)), 15µM 2-mercaptoethanol was added to the culture medium.

T cell isolation and activation

Murine $CD8^+$ T cells were purified from C57BL/6J or C57BL/6J.OT-I splenocytes by MACS-selection according to manufacturer's protocol (Miltenyi CD8 isolation kit; 90-99% purity) or FACS-sorting (BD FACSAria III Cell Sorter; > 99.9% purity). For *ex vivo* experiments, C57BL/6J $CD8\alpha\beta^+$ T cells were stimulated for 6 hours with 2µg/mL plate bound anti-CD3 alone, (17.A2, Bioceros), or in combination with 1µg/mL soluble anti-

110 CD28 (PV-1, Bioceros), 5μg/mL TLR2 ligand Pam_3CysSK_4 (Pam3) or 10μg/mL TLR7
111 ligand R848 (both InvivoGen).

112 For the generation of antigen-experienced T cells, 1×10^6 MACS-purified $CD8^+$ OT-I T
113 cells were activated for 20h MEC.B7.SigOVA cells as previously described (29).
114 Activated T cells were harvested, washed, and put to rest for 3-15 days in the presence of
115 10ng/mL recombinant murine IL-7 (rmIL-7; PeproTech). Resting OT-I T cells were
116 stimulated in serum-free IMDM for indicated time points with 0.1nM to 100nM OVA_{257-}
117 $_{264}$ peptide (GenScript), 5μg/mL Pam3, 10μg/mL R848, and/or 1ng/ml recombinant
118 murine IL-12 (PeproTech) in the presence of 1μg/mL brefeldin A (BD Biosciences)
119 and/or 2μM monensin (eBioscience) as indicated.

120

121 For studies with human T cells, peripheral blood mononuclear cells (PBMCs) were
122 isolated by Lymphoprep density gradient separation (Stemcell Technologies). $CD8^+$ T
123 cells were then purified from PBMCs by MACS-selection according to manufacturer's
124 protocol (Miltenyi CD8 isolation kit). $CD8^+$ T cells were stimulated in serum-free IMDM
125 for 6 hours with 1μg/mL plate-bound anti-CD3 alone (Hit3a, eBioscience), or in
126 combination with 1μg/mL soluble anti-CD28 (CD28.2, eBioscience), 0.1μg/mL Pam3 or
127 10μg/mL R848.

128

129 **B16 melanoma-T cell co-culture**

130 B16F10 melanoma cells were loaded with indicated amounts of $OVA_{257-264}$ peptide as
131 previously described (31). OT-I T cells were added to pre-seeded tumor cells for 5h at a

132 6:1 effector:target ratio. When indicated, 0.1, 1 or 5µg/mL Pam3 or 0.1, 1 or 10µg/mL
133 R848 was added at t=0h to the cultures.
134
135 **Flow cytometry and intracellular cytokine staining**
136 For flow cytometry analysis and sorting, cells were washed with FACS buffer
137 (phosphate-buffered saline [PBS], containing 1% FCS and 2mM EDTA) and labeled with
138 monoclonal antibodies with anti-CD8α (53-6.7), anti-CD8β (H35-17.2), anti-CD4 (clone
139 GK1.5), anti-CD44 (IM-7), anti-L-selectin (CD62L) (MEL-14), anti-CD11b (M1/70),
140 anti-CD11c (N418), anti-PD-L1 (clone MIH5), anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-
141 XT22), and anti-IL2 (JES6-5H4) (all from eBioscience) for mouse T cells, and with anti-
142 CD8 (SK1), anti-TNF-α (Mab11) (BD Biosciences), anti-IFN-γ (4.SB3), and anti-IL-2
143 (MQ1-17H12) (Biolegend) for human T cells. Near-IR (Life Technologies) was added to
144 exclude dead cells from analysis. For intracellular cytokine staining, cells were cultured
145 in the presence of 1µg/ml brefeldin A as indicated. For detection of degranulation, anti-
146 CD107a (eBio1D43) was added at t=0h to the culture. Upon stimulation, cells were fixed
147 and permeabilized with the Cytofix/Cytoperm kit according to the manufacturer's
148 protocol (BD Biosciences). The frequency of IFN-γ, TNF-α and IL-2 producing cells was
149 calculated as percentage of Near-IR^{neg} CD8α^{+} OT-I T cells, Near-IR^{neg} CD8αβ^{+} murine
150 T cells or Near-IR^{neg} CD8^{+} human T cells, unless otherwise specified. PD-L1 expression
151 was evaluated on NearIR^{neg} CD4^{+} CD8α^{-} B16-OVA cells. Expression levels were
152 acquired using FACS LSR Fortessa or FACSymphony (BD Biosciences) and data were
153 analyzed using FlowJo software (Tree Star, version 10).
154

155 **Single molecule FISH probes**

156 Single-molecule FISH probes of 20 nucleotides for *Ifng*, *Tnfa*, and *Il2* mRNA were
157 designed according to the manufacturer's guidelines (LGC Biosearch Technologies).
158 Probes with predicted high affinity for secondary target genes (identified by BLASTN)
159 were discarded when the murine gene skyline of the Immunological Genome Project
160 (*http://www.immgen.org*) indicated gene expression in T cells. This resulted in 33 probes
161 for *Ifng*, 45 probes for *Tnfa,* and 29 probes for *Il2*. Probes were Quasar 670-labeled.
162 Sequences are available upon request. Binding competition assays were performed with
163 identical unlabeled probe sets (Sigma-Aldrich). Quasar 670-labeled probes for human
164 *PHOX2B* were used as control probes.

165

166 **Flow-FISH**

167 The Flow-FISH protocol was adapted from (4). Briefly, T cells were activated with
168 indicated stimuli in the presence of 2µM monensin. Cells were stained for extracellular
169 markers, fixed, permeabilized and intracellular cytokine staining was performed with the
170 Cytofix/Cytoperm kit according to the manufacturer's protocol (BD Biosciences) in 96-
171 well V-bottom plates as previously described. All buffers, antibodies and reagents were
172 freshly supplemented with 4 units murine RNAse A/B/C inhibitor/ml prior to use (New
173 England BioLabs). Cells were washed twice with 200µl wash buffer (RNAse free water
174 containing 12.5% formamide (Sigma Aldrich), 2X SSC, and 4 units RNAse inhibitor/ml)
175 and transferred to 1.5 ml LoBind Eppendorf tubes (Eppendorf). Cells were incubated for
176 16h at 37°C + 5% CO_2 with 15nM Stellaris FISH probes in 50µl hybridization buffer
177 (RNAse free water containing 10% formamide, 1X SSC, 0.1 g/ml dextran sulfate salts

178 (Sigma Aldrich) and 40 units RNAse inhibitor/ml). Cells were washed once with 1 ml
179 wash buffer prior to acquisition in wash buffer by flow cytometry.
180
181 **Quantitative PCR analysis**
182 Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using
183 SuperScript III Reverse Transcriptase (Invitrogen) and quantitative Real-Time PCR was
184 performed with SYBR green and a StepOne Plus RT-PCR system (both Applied
185 Biosystems). Reactions were performed in duplicate or triplicate, and C_t values were
186 normalized to L32 levels. Primer sequences were previously described (29).
187 To determine the half-life of cytokine mRNA, T cells were activated for 3h with
188 indicated stimuli, and subsequently treated with 10µg/ml Actinomycin D (Sigma-
189 Aldrich) for indicated time points.
190
191 **Statistical analysis**
192 Results are expressed as mean ± SD. Statistical analysis between groups was performed
193 with GraphPad Prism 6, using paired or unpaired 2-tailed Student *t* test when comparing
194 2 groups, or 1-way or 2-way ANOVA test with Dunnett's or Tukey's multiple
195 comparison when comparing > 2 groups. P values < 0.05 were considered to be
196 statistically significant.

197 **Results**

198 **TLR2-mediated costimulation lowers the antigen threshold for cytokine production**
199 **of $CD8^+$ T cells**

200 We previously showed that TLR2 ligands can promote antigen-independent production of
201 IFN-γ in $CD8^+$ T cells (29). To determine whether TLR2 can also provide costimulatory
202 signals, we isolated and purified spleen-derived $CD8^+$ T cells from C57BL/6J mice which
203 were activated for 6h with αCD3 alone, or in combination with the TLR2 ligand
204 Pam_3CysSK_4 (Pam3). We measured the production of the three key cytokines that define
205 effective T cell responses, i.e. IFN-γ, TNF-α, and IL-2 (35-37).

206

207 The TLR2 ligand Pam3 potently increased the percentage of IFN-γ producing T cells
208 compared to αCD3 stimulation alone. In fact, Pam3 costimulation resulted in levels of
209 cytokine production similar to levels reached with αCD28 costimulation (Fig 1A).
210 Combining Pam3 and αCD28 induced even higher levels of cytokine production,
211 indicating that these two triggers acted synergistically (Fig 1A). Importantly, Pam3
212 costimulation was effective in MACS-purified (>90% purity) and in FACS-sorted
213 (>99.9% purity) $CD8^+$ T cells (Suppl Fig 1A-C), again demonstrating that the TLR2
214 ligand acted directly on the T cells (29). Whereas T cells triggered with Pam3 alone only
215 produce IFN-γ (29), they also produced more TNF-α and IL-2 when Pam3 was combined
216 with αCD3, or αCD3/αCD28 stimulation (Fig 1A, Suppl Fig 1B, C). In line with the
217 expression pattern of TLR2 (27, 29), Pam3 increased the cytokine production specifically
218 in memory-like $CD44^{hi}$ T cells, and not of $CD44^{low}$ naive T cells (Suppl Fig 1B, C).

219

220 To determine the effect of TLR2-mediated costimulation in combination with antigen-
221 specific activation, we turned to TCR transgenic OT-I T cells that were *in vitro* activated
222 and rested for 3-15 days in the absence of antigen as previously described (29). $CD8^+$ T
223 cell purity in this culture system reaches >99.9% at day 3 of culture ((29); data not
224 shown). Pam3 costimulation also significantly increased the cytokine production of OT-I
225 T cells activated with 100nM $OVA_{257-264}$ peptide (aa:SIINFEKL; N4; Fig 1B).
226 Intriguingly, T cells activated with the low affinity variant (aa:SIITFEKL; T4) in
227 combination with Pam3 produced comparable levels of cytokines as when T cells were
228 activated with the high affinity N4 peptide alone (Fig 1B). In line with these results,
229 when Pam3 was added to T cells activated with a low peptide concentration (0.1nM;
230 OVA_{low}), IFN-γ and TNF-α production raised from hardly detectable to significant levels
231 (Fig 1C). In addition, Pam3 substantially boosted the protein production of all three
232 measured cytokines in response to intermediate peptide concentrations (1nM; OVA_{int}; Fig
233 1C, data not shown). Thus, TLR2-mediated costimulation with Pam3 lowers the antigen
234 threshold required for T cell activation.

235

236 Human T cells also express TLR2, which is upregulated upon TCR triggering (27). To
237 determine whether the costimulatory capacity of TLR2 ligands was conserved between
238 mice and men, we purified $CD8^+$ T cells from human PBMCs and stimulated them for 6h
239 with αCD3 alone, or in combination with TLR2 ligand Pam3. Pam3 potently increased
240 the percentage of IFN-γ-producing human $CD8^+$ T cells to levels of cytokine production
241 that were comparable to CD28-mediated costimulation (Fig 1D). Altogether, our findings

242 demonstrate that the TLR2 ligand Pam3 provides costimulatory signals to both murine
243 and human T cells.
244
245 **Simultaneous analysis of cytokine mRNA and protein production of murine T cells**
246 We next questioned which signals drive the cytokine production of T cells. At the
247 molecular level, the integration of transcriptional and post-transcriptional regulatory
248 events is required to coordinate the production of cytokine (31, 38-42). To dissect the link
249 between distinct T cell signals and regulation of cytokine production, the analysis of both
250 mRNA and protein levels is thus warranted. Because $CD8^+$ T cells respond
251 heterogeneously to activation (3, 4, 31), the analysis of bulk populations can however
252 mask differences to various stimuli. This may be of particular importance when
253 measuring the effect of costimulatory signals. We therefore reasoned that measuring
254 mRNA and protein on a single cell level would help to better define T cell responses (4,
255 43).
256
257 We recently established a multi-color flow-cytometry-based fluorescence *in situ*
258 hybridization (Flow-FISH) protocol for human T cells that allowed us to simultaneously
259 detect cytokine mRNA and protein with a single-cell resolution (4). Here, we optimized
260 Flow-FISH for mouse T cells (Fig 2A). Upon T cell activation with 100nM OVA
261 (OVA_{hi}), all three cytokine mRNAs and corresponding proteins were detectable (Fig 2A).
262 The mRNA signal was specific for all three cytokines as determined with an unrelated
263 FISH probe set directed against human *PHOX2B* mRNA (control; Fig 2A). Furthermore,

264 when we spiked in increasing amounts of unlabeled cytokine probes to activated T cells,
265 the mRNA signal decreased in a concentration-dependent manner (Suppl Fig 2).
266
267 We next determined the sensitivity of Flow-FISH. To this end, we turned to our previous
268 findings that cytokine mRNA levels do not always correlate with the actual protein
269 output. The production of TNF-α predominately depends on translation of pre-formed
270 mRNA, and only high antigen levels also drive *de novo* mRNA transcription. In contrast,
271 the production of IL-2 depends entirely on *de novo* transcription, whereas IFN-γ
272 production depends both on *de novo* transcription and on translation of pre-formed
273 mRNA (31). To determine the capacity of Flow-FISH to detect these differences, we
274 activated T cells with different amounts of antigen and followed their responsiveness
275 over time. As expected, the overall percentage of $mRNA^+$ and $protein^+$ T cells with Flow-
276 FISH was highest when T cells were stimulated with OVA_{hi} (Fig 2B). Furthermore,
277 Flow-FISH revealed a cytokine-specific ratio between $mRNA^+$ and $protein^+$ T cells (Fig
278 2B). In line with the predominant role of translation over transcription for TNF-α
279 production (31), the percentage of *Tnfa* $mRNA^+$ cells was always lower than that of
280 TNF-α $protein^+$ T cells (Fig 2B, C). In contrast, irrespective of the antigen load, *Il2*
281 mRNA levels always matched with the corresponding protein levels (Fig 2B, C). For
282 IFN-γ, the kinetics of mRNA and protein overlapped when T cells were stimulated with
283 OVA_{int}, and activation with OVA_{hi} resulted in higher levels of $protein^+$ than of $mRNA^+$
284 cells (Fig 2C). These findings show that Flow-FISH is an effective method to visualize
285 the relation of cytokine mRNA and protein on a single cell level.
286

287 **TLR2 triggering boosts *Ifng* mRNA transcription**

288 Cytokines follow individual production kinetics upon T cell activation with antigen (3, 4,
289 31). To determine if costimulation through TLR2 triggering altered the magnitude and/or
290 the kinetics of cytokine production, we measured IFN-γ, TNF-α and IL-2 protein by
291 intracellular cytokine staining. To follow cytokine production over time, we only blocked
292 protein secretion with brefeldin A for the last 2h of stimulation. Pam3 significantly
293 enhanced the percentage of IFN-γ, TNF-α and IL-2 producing T cells upon OVA_{int}
294 stimulation, with the most prominent effect on IFN-γ (Fig 3A). Pam3 mediated
295 costimulation did not affect the kinetics of TNF-α and IL-2 production. Interestingly, the
296 levels of IFN-γ production after 4h of OVA_{int} stimulation together with Pam3 equaled
297 those of OVA_{hi} stimulation (Fig 3A), but with a significant delay of the onset of
298 production at 2h (OVA_{int}+Pam3= 7±2% IFN-γ^+ T cells, compared to OVA_{hi}= 30±8%
299 IFN-γ^+ T cells). Thus, Pam3 primarily boosts the production of IFN-γ, albeit with a delay
300 in the response kinetics.

301

302 We previously found that the TLR2-mediated innate IFN-γ production depends on *de*
303 *novo* mRNA transcription (29). To determine if similar mechanisms apply for Pam3-
304 mediated costimulation we first measured the cytokine mRNA levels with Flow-FISH.
305 The *Ifng* mRNA levels were not significantly higher in T cells activated for 2h with
306 OVA_{int} with or without Pam3 costimulation (Fig 3B). At 4h and 6h of activation,
307 however, the addition of Pam3 significantly boosted the *Ifng* mRNA levels to a similar
308 magnitude as upon stimulation with OVA_{hi} (Fig 3B). Flow-FISH thus showed that TLR-

309 2-mediated costimulation enhances cytokine production by maintaining high mRNA
310 levels.
311
312 **TLR2 triggering amplifies the TCR signals to enhance *Ifng* mRNA stability**
313 The magnitude and the duration of IFN-γ production depends on the capacity to stabilize
314 *Ifng* mRNA (31). Indeed, increased antigen load stabilizes the *Ifng* mRNA (Fig 4A). We
315 determined the mRNA half-life by measuring the *Ifng* mRNA decay from 3h of
316 activation onwards, with RNA polymerase II transcription inhibitor Actinomycin D
317 (ActD). In line with the limited cytokine production (Fig 1C), activation with OVA_{low}
318 displayed an equally short *Ifng* half-life as unstimulated T cells (t1/2= ~30 min; Fig 4A;
319 (29)). Stimulation with OVA_{int} and OVA_{hi}, however, significantly prolonged the half-life
320 of *Ifng* in a dose-dependent manner (OVA_{int}: t1/2= ~90 min, OVA_{hi}: t1/2 > 2h; Fig 4A).
321
322 It was previously shown that costimulation through CD28 and LFA-1 stabilizes cytokine
323 mRNA (32, 33). Stabilization of *Ifng* mRNA was also observed upon Pam3
324 costimulation. T cell activation with OVA_{low} plus Pam3 increased *Ifng* mRNA half-life to
325 levels similar to OVA_{int} stimulation (t1/2= ~90 min, compare Fig 4A with Fig 4B, left
326 panel). Similarly, OVA_{int} plus Pam3 significantly enhanced *Ifng* mRNA stability (t1/2 >
327 2h, Fig 4B, right panel).
328
329 Because T cell activation with Pam3 alone did not stabilize the *Ifng* mRNA (29), we
330 hypothesized that costimulation itself may not be the determinant factor inducing mRNA
331 stabilization. Rather, we postulated that TCR signaling is required for mRNA stability,

332 and that costimulation can potentiate this process. To study this hypothesis, we activated
333 T cells in a fully antigen-independent manner. When Pam3 stimulation was combined
334 with IL-12, we measured IFN-γ protein levels that were similar to activation with OVA_{int}
335 (Fig 4C, D left panel). Interestingly, at 2h of stimulation Pam3 plus IL-12 induced higher
336 *Ifng* mRNA levels than stimulation with OVA_{int} (Fig 4D right panel). The accumulation
337 of *Ifng* mRNA, however, could not be attributed to stabilization of mRNA (Fig 4E). In
338 conclusion, TLR2 triggering only stabilizes *Ifng* mRNA in the presence of TCR
339 engagement.
340

341 **TLR7-mediated costimulation supports the production of IFN-γ without mRNA**
342 **stabilization**

343 Not only TLR2 triggering, but also TLR7 triggering induces IFN-γ production by T cells
344 in an antigen-independent manner (29). To determine if TLR7 ligands also provide
345 costimulatory signals, we stimulated OT-I T cells with OVA_{low} or OVA_{int} in combination
346 with the TLR7 ligand R848. As TLR2 engagement alike, TLR7 triggering lowered the
347 antigen threshold of T cell activation and significantly increased the production of IFN-γ
348 (Fig 5A). Similar results were found for *ex vivo* FACS-sorted memory-like $CD44^{hi}$ $CD8^{+}$
349 T cells activated with αCD3 and/or αCD28 and R848 (Suppl Fig 1D). However, in
350 contrast to Pam3, this effect appears to be species-specific, because R848 does not
351 enhance the production of IFN-γ of human $CD8^{+}$ T cells (Suppl Fig 1E).
352 When turning back to our mouse system, we observed that R848 also supported IL-12-
353 mediated innate production of IFN-γ (Fig 5A). The increase in IFN-γ protein production
354 directly correlated with increased *Ifng* mRNA levels (Fig 5B). *Ifng* mRNA consistently

355 displayed a short half-life when T cells were stimulated in an antigen-independent
356 manner (compare Fig 4E and Fig 5C), resulting in similar levels of protein production
357 when Pam3 or R848 were used in combination with IL-12 (Fig 5D). Interestingly, TLR7-
358 mediated costimulation completely failed to prolong *Ifng* mRNA half-life when engaged
359 in combination with the TCR (Fig 5C), and led to lower levels of protein production than
360 TLR2-mediated costimulation (Fig 5D). Altogether, our data show that the benefit of
361 mRNA stabilization requires TCR signaling, can be prolonged by TLR2 ligands and
362 significantly potentiates cytokine production.
363

364 **TLR2-mediated costimulation supports polyfunctional CD8$^+$ T cell responses**
365 **against tumor cells**

366 TLR ligands are often used as adjuvants for immunotherapy to boost innate immune
367 responses and enhance antigen presentation (44-51). We here investigated whether TLR
368 ligands can also directly augment the cytokine profile of T cell responses against tumor
369 cells. We loaded B16F10 melanoma cells with increasing amounts of $OVA_{257-264}$ peptide,
370 and measured the production of IFN-γ, TNF-α and IL-2 by OT-I T cells after 5h of co-
371 culture with the tumor cells, in the presence or absence of Pam3 or R848. Whereas TLR
372 costimulation increased the production of TNF-α and IL-2 only at high antigen levels, the
373 production of IFN-γ was also supported at low amounts of antigen (Fig 6A, Suppl Fig
374 3A). The effects of TLR-mediated costimulation were dose-dependent. Increasing
375 concentrations of TLR ligands potentiated the overall percentage of cytokine-producing T
376 cells in response to B16-OVA cells that constitutively expressed the C-terminal part of
377 ovalbumin (32) (Fig 6B). Interestingly, the expression of the degranulation marker

378 CD107a did not change upon TLR-mediated costimulation (Suppl Fig 3B), indicating
379 that TLR signaling specifically promoted cytokine production.
380
381 Potent effector T cells produce two or more cytokines (1-3). This polyfunctional profile
382 correlates with a higher efficacy of vaccines and T cell therapies (5, 36). Intriguingly,
383 even though Pam3 and R848 both supported the cytokine production of T cells in
384 response to tumor cells, the polyfunctional profile of effector T cells was only enhanced
385 by TLR2 ligand Pam3 (Fig 6C). Higher doses of R848 skewed the immune response
386 towards single IFN-γ-producing T cells (Fig 6C). Of note, TLR ligands alone did not
387 affect cell viability of tumor cells, or the T-cell mediated induction of PD-L1 and MHC-
388 II expression (Suppl Fig 3C, D and data not shown). In conclusion, TLR2 and TLR7
389 ligands provide costimulatory signals to $CD8^+$ T cells, but they do so through different
390 mechanisms. Importantly, only TLR2 triggering promotes polyfunctional $CD8^+$ T cell
391 responses.

392 **Discussion**

393 $CD8^+$ T cells receive different stimuli upon infection that shape their effector functions.
394 This allows T cells to appropriately respond to specific pathogens. Here, we show that
395 TLR2 and TLR7 provide costimulatory signals to amplify the magnitude of cytokine
396 production in murine and in human $CD8^+$ T cells. TLR ligands lower the antigen
397 threshold required for T cell activation independently of antigen avidity and/or affinity
398 for the TCR. Whereas antigen-independent engagement of TLRs only induces the
399 production of IFN-γ (29), in combination with TCR triggering, TLR-mediated
400 costimulation also supports the production of TNF-α and IL-2.

401

402 In agreement with our previous findings (29), TLR signaling significantly promotes the
403 transcription of *Ifng* mRNA. This effect is further enhanced when TLR ligands synergize
404 with suboptimal antigen levels or with antigen-independent stimuli, such as IL-12. The
405 exact mode of action of TLR-mediated support of TCR and IL-12 signaling is not yet
406 known, and several signal-transduction pathways can be involved. We and others
407 previously showed that TLR2-dependent transcription of *Ifng* mRNA in T cells engages
408 PI3K and Akt signaling (29, 52). In dendritic cells and macrophages, TLR2 and TLR7
409 engagement also activates nuclear factor kappa B (NF-κB) (53, 54) and activator protein
410 1 (AP-1) (55, 56), two crucial transcription factors for cytokine production (57, 58).
411 Because PI3K/Akt, NF-κB and AP-1 are also activated through the TCR, we hypothesize
412 that TLR-mediated costimulation can further amplify these pathways in T cells, and
413 synergize with the TCR-dependent transcription factor NFAT to maximize *Ifng* mRNA
414 transcription (57). Interestingly, NF-κB and AP-1 can also be activated downstream of

415 the IL-18 receptor and synergize with IL-12-mediated STAT4 activity to enhance
416 antigen-independent *Ifng* gene expression in T cells (59-61). It is therefore tempting to
417 speculate that the engagement of NF-κB and AP-1 represents the main mode of action of
418 TLR ligands to boost *Ifng* mRNA transcription in T cells, irrespective from the presence
419 of the antigen.
420
421 *De novo* transcription of cytokine mRNA is however not sufficient to optimally shape the
422 magnitude and kinetics of cytokine production (31). mRNA turnover is another critical
423 parameter (38-41), and the stability of *Ifng* mRNA strongly depends on the quality and
424 the quantity of stimuli that a T cell receives (29, 31). T cell activation through TLRs
425 alone fails to stabilize *Ifng* mRNA, thereby restricting the magnitude of innate-like
426 cytokine production by T cells (29). Similarly, suboptimal antigen levels alone cannot
427 promote the stabilization of *Ifng* mRNA, but it can be induced with additional
428 costimulatory signals. As for classical costimulatory molecules alike (32, 33), we show
429 here that TLR2 signaling synergized with TCR signaling to stabilize *Ifng* mRNA.
430 Interestingly, TLR7 costimulation failed to stabilize *Ifng* mRNA. The triggering of TLR2,
431 but not TLR7, activates the mitogen-activated protein kinases (MAPK) cascade (54), and
432 MAPK signaling can enhance cytokine mRNA stability (62, 63). Whether TLR2 ligands
433 support TCR-mediated stabilization of cytokine mRNA through MAPK signaling
434 remains to be determined. Regardless of the mechanism, costimulation through TLR2
435 potentiates cytokine production in response to suboptimal antigen loads by enhancing
436 TCR-mediated stabilization of *Ifng* mRNA.
437

438 TLR ligands are currently used as adjuvants for vaccines and T cell therapies (44-51). In
439 addition to their well-known effect in boosting innate immune responses and enhancing
440 antigen presentation, we reveal that TLR ligands can also directly augment T cell
441 responses. We recently showed that tumor-infiltrating T cells lose their capacity to
442 produce cytokines because they fail to stabilize the cytokine mRNA (Salerno et al.,
443 BioRxiv, 339580). It is therefore tempting to speculate that TLR2 could promote anti-
444 tumoral T cell responses by providing costimulatory signals to T cells and prolonging
445 cytokine mRNA half-life. This in turn can potentiate the response rate of other immune
446 cell types, as shown for B cells and macrophages (29, 64). TLR ligands may also act on T
447 cell subsets residing at barrier tissues, like tissue resident memory T cells. These cells are
448 routinely exposed to danger signals and non-cognate pathogens, and may thus develop
449 cell-specific regulatory mechanisms. In conclusion, dissecting how TLR signals integrate
450 within T cells to promote optimal cytokine production may be crucial to rationalize the
451 use of TLR ligands as adjuvants and improve the efficacy of vaccines and
452 immunomodulatory therapies.

453 **Author Contributions**

454 F.S., J.J.F.H., and A.G. performed experiments, F.S., J.J.F.H., and M.C.W. designed
455 experiments, analyzed data, and wrote the manuscript.

456

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460

461 **Disclosures**

462 The authors have no financial conflict of interest.

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666

667 **FIGURE LEGENDS**

668 **Figure 1: TLR2 acts as a costimulatory molecule and enhances cytokine production**
669 **of murine and human $CD8^+$ T cells**

670 (**A**) $CD8^+$ T cells purified from C57BL/6J mice were *ex vivo* stimulated with 2μg/ml
671 αCD3, with or without 5μg/ml TLR2 ligand Pam_3CysSK_4 (Pam3), 1μg/ml αCD28, or a
672 combination thereof. Dot plots represent IFN-γ and TNF-α production measured by
673 intracellular cytokine staining. For pooled data see Suppl Fig 1B. (**B-C**) *In vitro* activated
674 $CD8^+$ OT-I T cells were cultured in rmIL-7 for 3-15 days. T cells were then reactivated
675 with 100nM SIINFEKL (N4) or SIITFEKL (T4) $OVA_{257-264}$ peptide (B), or with 0.1nM
676 (low) or 1nM (int) SIINFEKL $OVA_{257-264}$ peptide (C). When indicated, 5μg/ml Pam3 was
677 added to the cell culture. (B) Compiled data (n=6 mice±SD) of percentage of cytokine
678 producing T cells from two independently performed experiments [Paired Student *t*-test;
679 *p<0.05; **p<0.01; ****p<0.0001]. (C) Representative dot plots of 8 mice and 4
680 independently performed experiments. (**D**) Left: dot plots represent IFN-γ and TNF-α
681 production of human $CD8^+$ T cells that were stimulated with 1μg/ml αCD3, with or
682 without 0.1μg/ml TLR2 ligand Pam3 or 1μg/ml αCD28. Right: compiled data (n=6
683 donors±SD) of percentage of IFN-γ producing T cells from two independently performed
684 experiments [RM-ANOVA with Dunnett's multiple comparissons collection; *p<0.05].
685 (A-E) T cells were cultured for 6h in the presence of Brefeldin A. T cells cultured in the
686 absence of stimuli were used as negative control.

687

688 **Figure 2: Simultaneous detection of cytokine mRNA and protein by Flow-FISH of**
689 **murine T cells**
690 (**A**) $CD8^+$ OT-I T cells (rested for 5-7 days) were stimulated for 4h with 100nM OVA
691 peptide, and cytokine mRNA and protein expression was analyzed by flow cytometry. T
692 cells were stained with FISH probes specific for *Ifng*, *Tnfa* and *Il2* mRNA (upper panel),
693 or with an unrelated FISH probe set against human *PHOX2B* (lower panel). Dot plots are
694 representative of at least 2 independently performed experiments. (**B-C**) *Ifng*, *Tnfa* and
695 *Il2* mRNA expression and IFN-γ, TNF-α and IL-2 protein production of untreated T cells
696 or T cells stimulated with 1nM (OVA_{int}) or 100nM (OVA_{hi}) OVA peptide for indicated
697 time points. (B) Dot plots represent cytokine production at 4h of activation. (C) Graphs
698 (mean±SD) show percentage of total $mRNA^+$ and total $protein^+$ T cells during the entire
699 time course. Data are representative (B) or pooled (C) from 3 independently performed
700 experiments.
701
702 **Figure 3: TLR2-mediated costimulation augments *Ifng* mRNA expression levels**
703 (**A**) Resting OT-I T cells were stimulated with OVA_{int}, OVA_{int} plus Pam3 or OVA_{hi} for
704 indicated time points. 1μg/ml brefeldin A was added during the last 2h of activation and
705 intracellular cytokine staining was performed to analyze protein production kinetics.
706 Graphs indicate the total percentage of IFN-γ^+, TNF-α^+ and IL-2^+ T cells (mean±SD)
707 pooled from 3 mice and 3 independently performed experiments. (**B**) *Ifng* mRNA
708 expression and IFN-γ protein production was determined by Flow-FISH upon 2, 4, or 6h
709 T cell activation as described above. Unstimulated T cells were used as negative control.

710 Graph indicates the total percentage of *Ifng* mRNA$^+$ T cells (mean±SD) pooled from 5
711 mice and 4 independently performed experiments.
712
713 **Figure 4: TLR2 triggering enhances TCR-dependent *Ifng* mRNA stability**
714 (**A-B**) OT-I T cells were stimulated for 3h with OVA_{low}, OVA_{int} or OVA_{hi} (A), or with
715 OVA_{low} or OVA_{int} in combination with Pam3 (B), and then incubated with 10μg/ml
716 Actinomycin D (ActD). T cells were harvested at the indicated time points to determine
717 *Ifng* mRNA stability by RT-PCR. Graphs show data (mean±SD) pooled from 6-8 mice
718 and 4 independently performed experiments [one-way ANOVA with Dunnett's multiple
719 comparison; *p<0.05; **p<0.01; ***p<0.001]. (**C**) Intracellular IFN-γ staining of OT-I T
720 cells stimulated for 6h with 5μg/ml Pam3, 1ng/ml rIL-12, or a combination thereof.
721 Unstimulated T cells were used as negative control. For pooled data see panel D. (**D**) T
722 cells were stimulated with OVA_{low}, OVA_{int} or IL-12, with or without the addition of
723 Pam3. Left: graph depicts the percentage of IFN-γ producing T cells after 6h stimulation.
724 Right: *Ifng* mRNA expression was measured by RT-PCR upon 2h T cell stimulation.
725 Data were pooled from 3 independently performed experiments (n=4-5 mice; mean±SD)
726 [Unpaired Student *t*-test; **p<0.005; ***p<0.0005; ****p<0.0001]. (**E**) *Ifng* mRNA
727 stability of T cells stimulated for 3h as indicated. Data pooled from 4 mice and two
728 independently performed experiments (mean±SD) [one-way ANOVA with Dunnett's
729 multiple comparison; ns= non-significant].
730
731 **Figure 5: The engagement of TLR7 promotes IFN-γ production of T cells without**
732 **enhancing *Ifng* mRNA stability**

733 T cells were stimulated with OVA_{low}, OVA_{int} or IL-12, with or without the addition of
734 10μg/ml R848. Unstimulated T cells were used as negative control. (**A**) Intracellular IFN-
735 γ staining of T cells stimulated for 6h. For pooled data see panel B. (**B**) Top: IFN-γ
736 protein production. Bottom: *Ifng* mRNA expression of T cells stimulated for 2h, and
737 measured by RT-PCR. Data were pooled from 3 independently performed experiments
738 (n=4-5 mice; mean±SD) [Unpaired Student *t*-test; *p<0.05; **p<0.005; ***p<0.0005].
739 (**C**) *Ifng* mRNA stability was determine upon 3h T cell activation, and addition of ActD
740 for indicated time points. Data were pooled from 4 independently performed experiments
741 (n=6-8 mice; mean±SD). (**D**) IFN-γ protein production after 6h T cell stimulation with
742 indicated stimuli. Graphs show data (mean±SD) pooled from 6 mice and 3 independently
743 performed experiments [one-way ANOVA with Tukey's multiple comparison; *p<0.05;
744 **p<0.01].
745
746 **Figure 6: TLR-triggering promotes polyfuntional T cell responses to tumor cells**
747 (**A**) OT-I T cells were co-cultured at a 6:1 effector:target ratio with B16F10 melanoma
748 cells loaded with indicated amounts of $OVA_{257-264}$ peptide for 6h. When specified, 5μg/ml
749 Pam3 or 10μg/ml R848 were added to the culture system. Graphs represent IFN-γ, TNF-
750 α and IL-2 production of OT-I T cells measured by intracellular cytokine staining. Data
751 is representative of 3 independently performed experiments (n=6 mice; mean ± SD) [two-
752 way ANOVA with Dunnett's multiple comparison; *p<0.05; **p<0.01]. For
753 representative dot plots, see Suppl Fig 3A. (**B-C**) OT-I T cells were co-cultured with B16
754 melanoma cells constitutively expressing the C-terminal part of ovalbumin (B16-OVA),
755 as described above. Increasing amounts of Pam3 or R848 were added as indicated. (B)

756 Dot plots represent IFN-γ and TNF-α production measured by intracellular cytokine
757 staining at 5h of activation. (C) Graphs depict the cytokine profile analysis of T cells
758 activated as in B. Data are representative (B) or pooled (C) from 6 mice and three
759 independently performed experiments (mean ± SD).

760 **SUPPLEMENTARY FIGURE LEGENDS**

761 **Supplementary Figure 1: Cytokine production of activated CD8$^+$ T cells**

762 (**A**) Dot plots represent CD8αβ$^+$ T cells (left) and CD8β$^-$CD11b$^+$ and CD8β$^-$CD11c$^+$
763 myeloid cells (right) before selection, after MACS-selection, or after FACS-sorting. (**B-**
764 **D**) MACS-selected (B) or FACS-sorted (C-D) murine CD8αβ$^+$ T cells were stimulated
765 for 6h with Pam3 (B-C), or R848 (D), in combination with anti-CD3 alone or
766 αCD3/αCD28. IFN-γ, TNF-α and IL-2 production was measured by intracellular
767 cytokine staining. Graphs depict the percentage (mean±SD) of cytokine producing
768 CD44^{hi}CD8αβ$^+$ T cells (left) or CD44^{low}CD8αβ$^+$ T cells (right) (n=2-3 mice) (**E**)
769 Purified human CD8$^+$ T cells were stimulated for 6h with αCD3 alone, or in combination
770 with αCD28, Pam3 (left), or R848 (right). Graphs depict the percentage (mean±SD) of
771 cytokine producing CD8$^+$ T cells. Data ± SD are pooled from 6 donors and two
772 independently performed experiments.

773

774 **Supplementary Figure 2: Validation of Flow-FISH analysis of murine CD8$^+$ T cells**

775 Resting OT-I T cells were stimulated for 4h with 100nM OVA peptide, and cytokine
776 mRNA and protein expression was measured by Flow-FISH. The specificity of *Ifng*,
777 *Tnfa*, and *Il2* FISH probe signal was determined by adding increasing amounts of
778 competing unlabeled probes during the staining procedure.

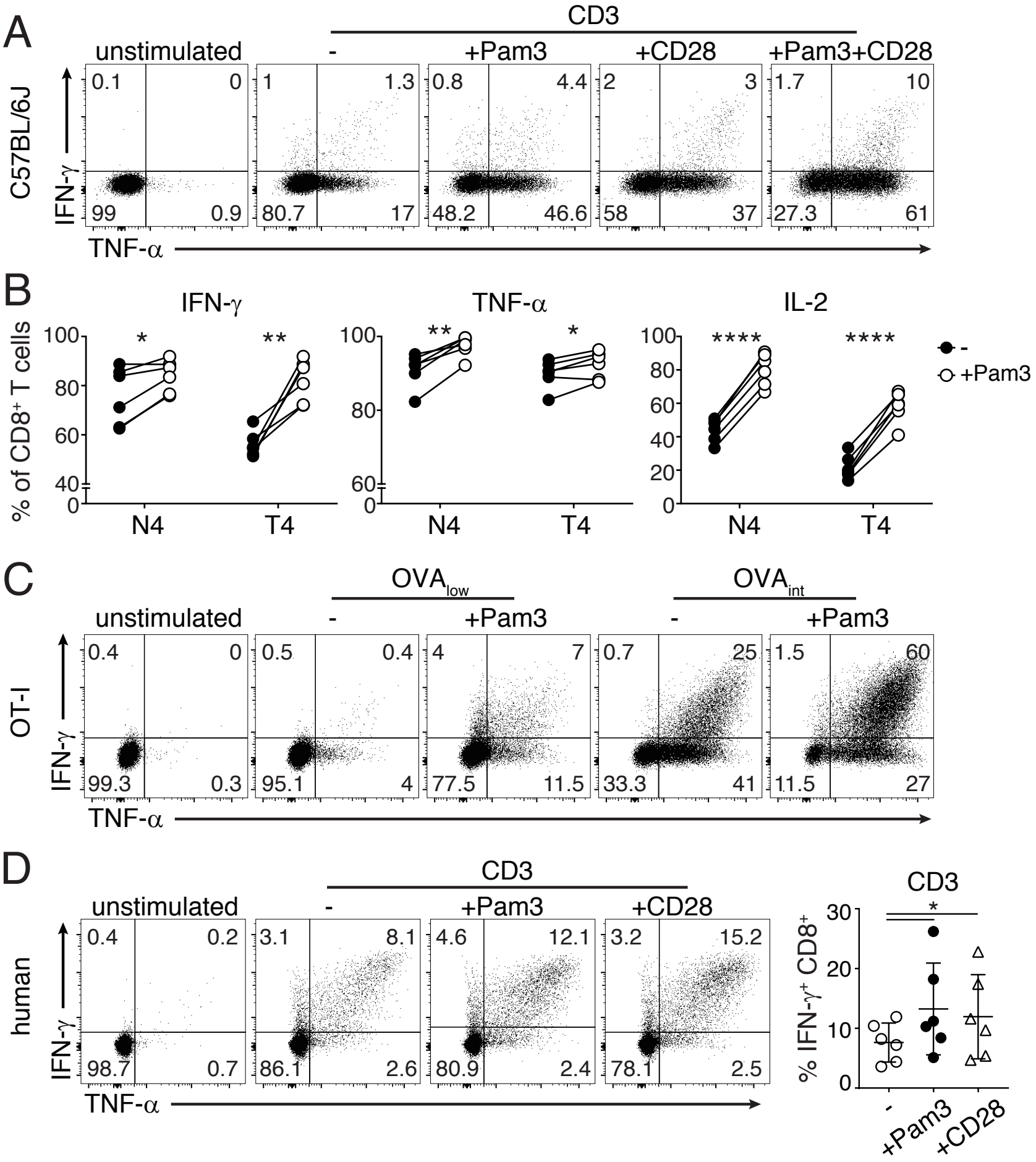
779

780 **Supplementary Figure 3: Cytokine production of OT-I T cells upon co-culture with**
781 **B16 melanoma cells and the effect of TLR-ligands on B16-OVA cells.**

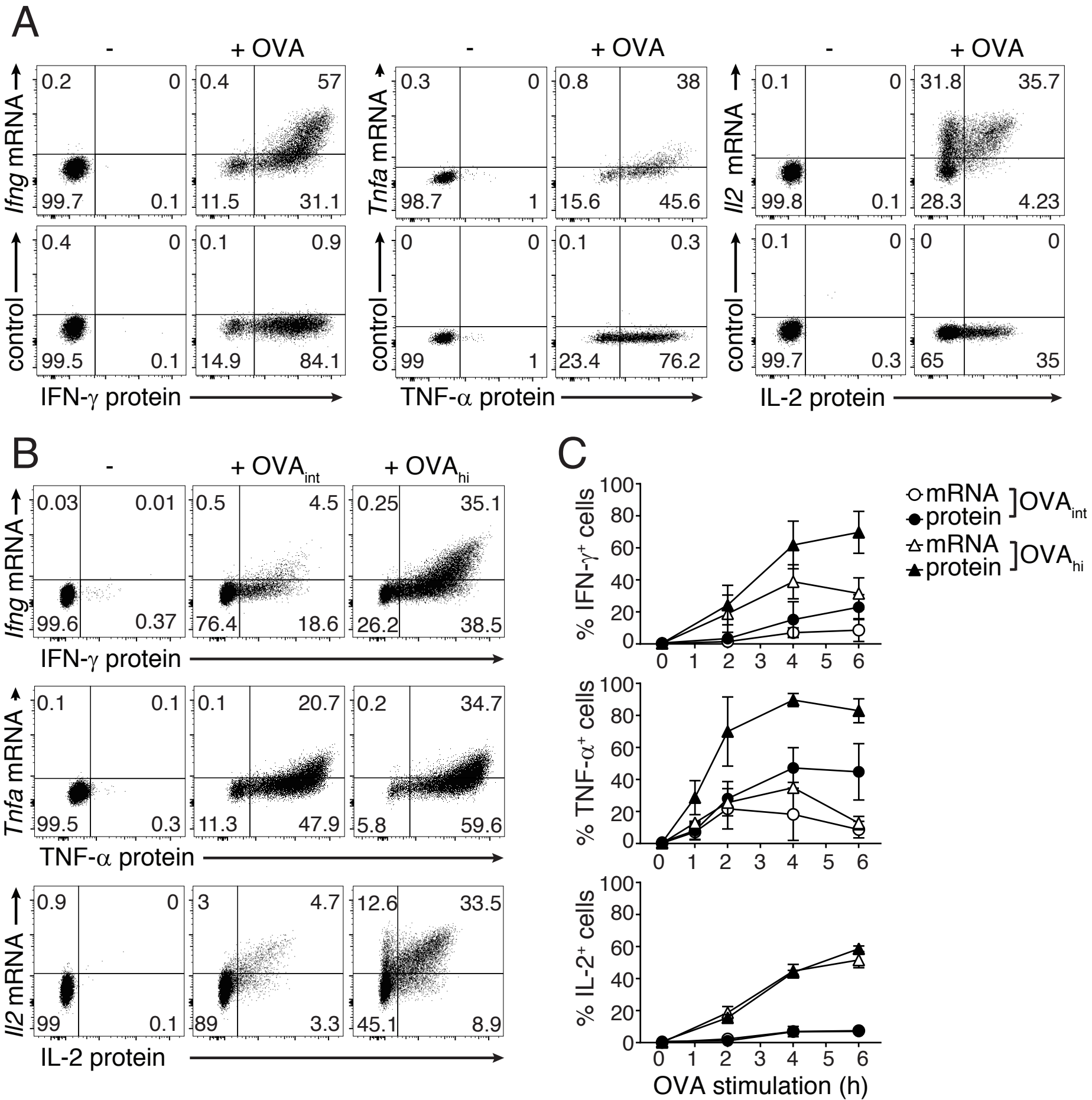
782 (**A**) Representative dot plots of IFN-γ and TNF-α production of OT-I T cells that were
783 co-cultured for 5h with B16F10 melanoma cells loaded with 0.1nM (low), 1nM (int) or
784 100nM (hi) $OVA_{257-264}$ peptide, with or without 5μg/ml Pam3 or 10 μg/ml R848. (**B**)
785 Graph depicts the percentage (mean±SD) of $CD107a^+$ $CD8^+$ OT-I T cells that were co-
786 culture for 5h with B16-OVA cells with or without the presence of increasing amounts of
787 Pam3 or R848 (n=4-8 mice). (C) Graph indicates the percentage of live B16-OVA cells,
788 defined as Near-IR^- $CD4^+$, that were cultured for 5h in the only presence of Pam3 or
789 R848. Data are pooled from 3 independently performed experiments (mean±SD). (D)
790 PD-L1 expression was measured on B16-OVA cells that were cultured alone (shaded
791 black histograms) or co-cultured with OT-I T cells (shaded red histograms), in the
792 presence of indicated amounts of Pam3 or R848. Numbers indicate PD-L1 Geo-MFI.
793 Data are representative of two independently performed experiments.

794

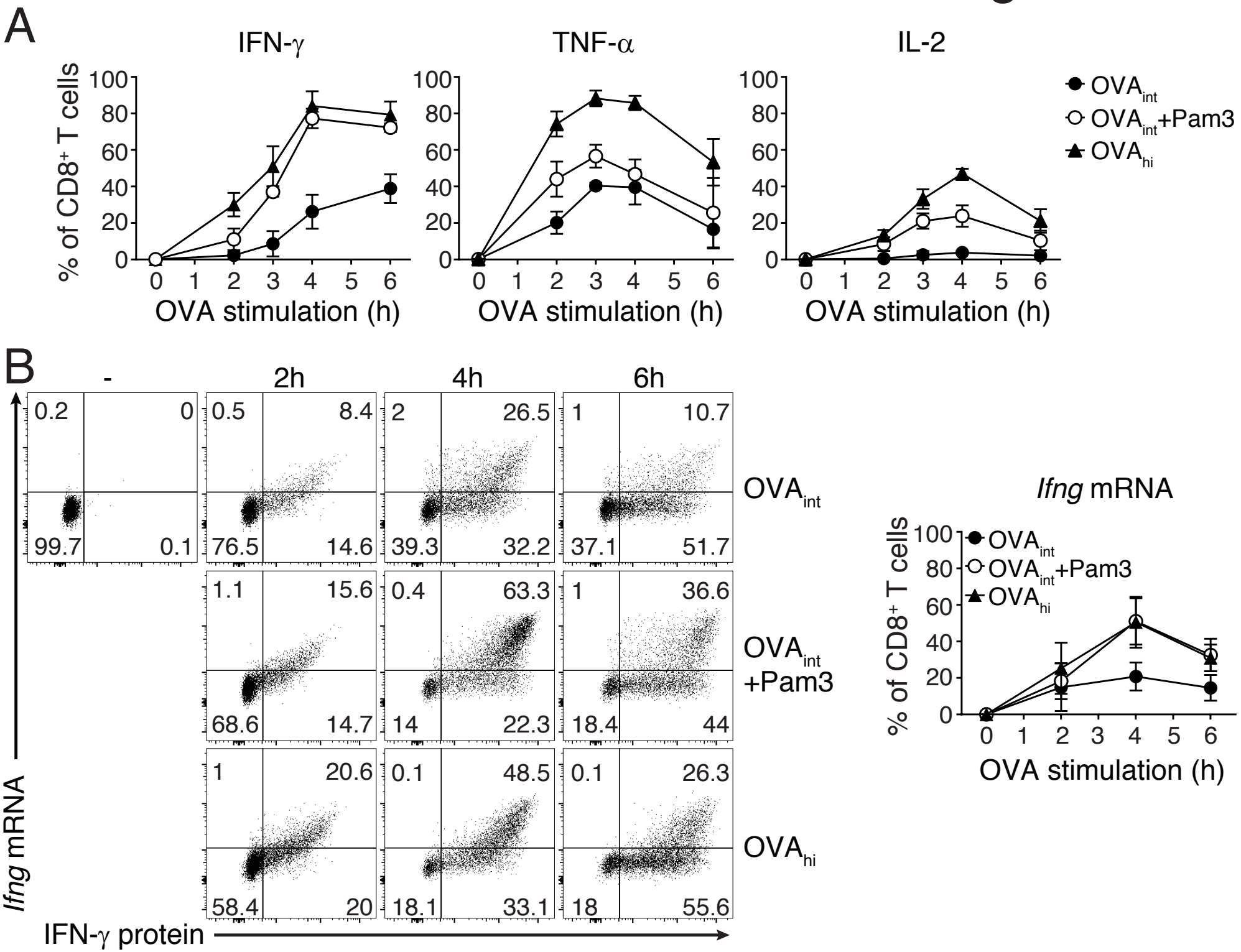
Salerno, Freen-van Heeren *et al.* Figure 1



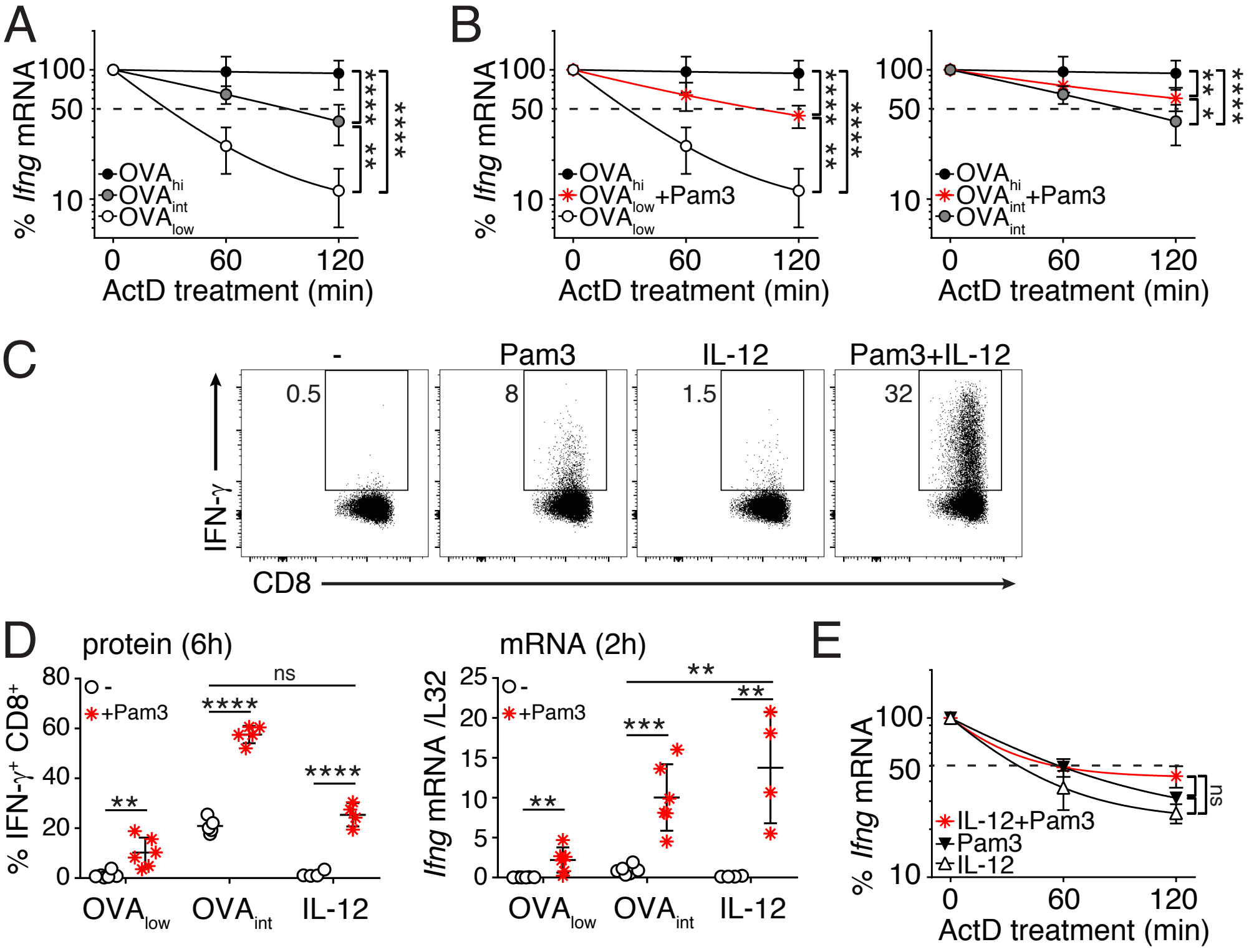
Salerno, Freen-van Heeren *et al.* Figure 2



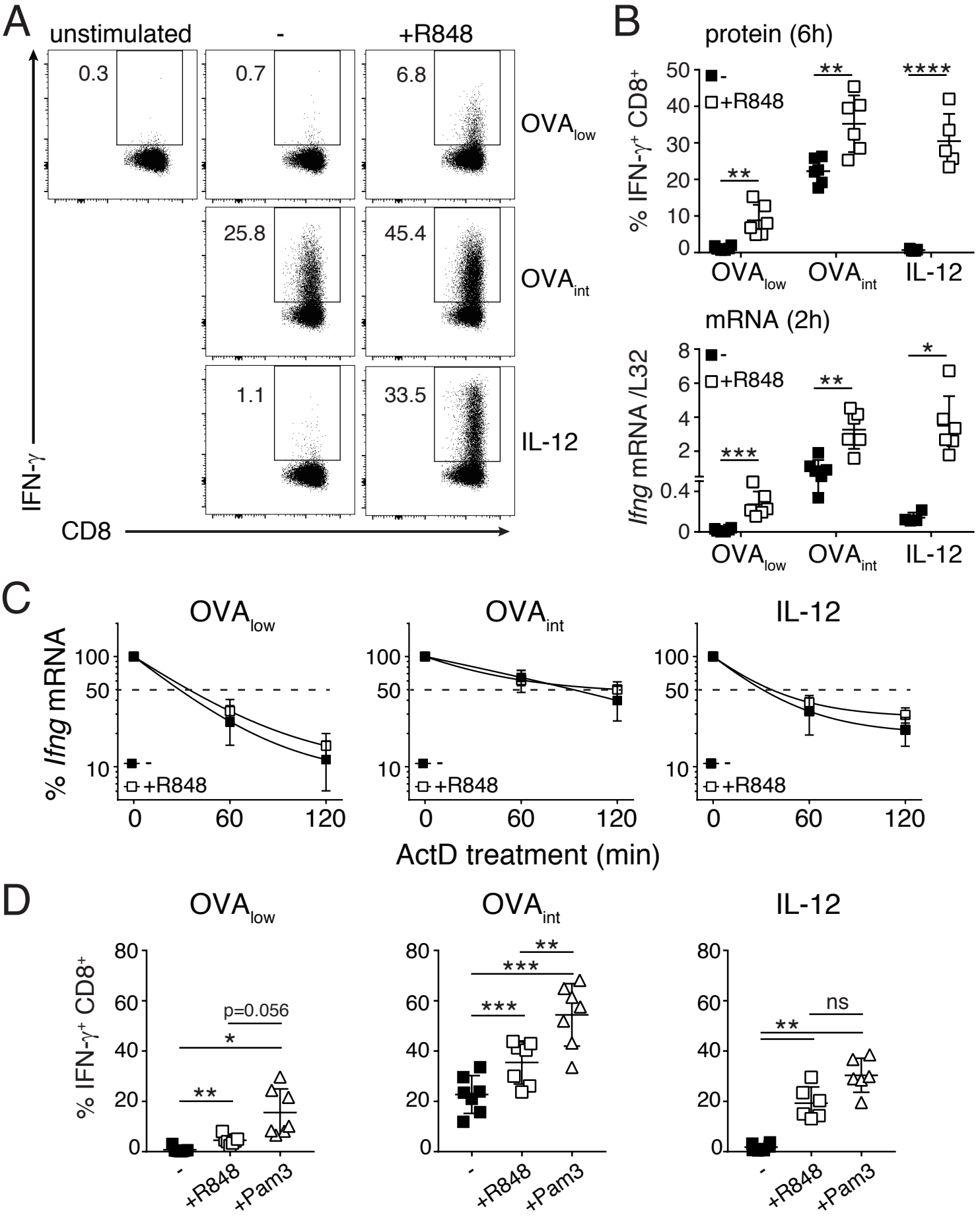
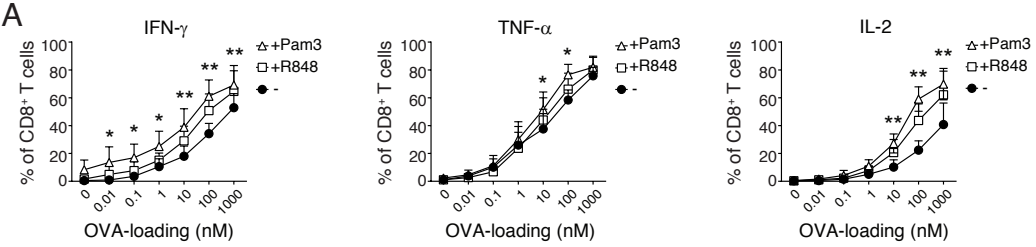
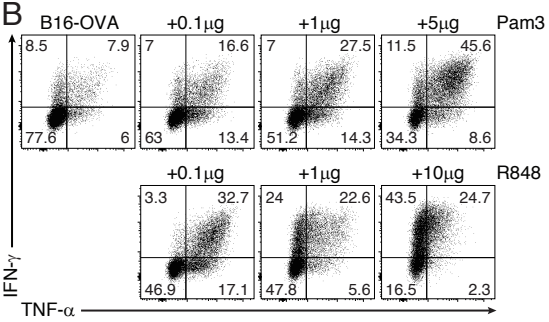
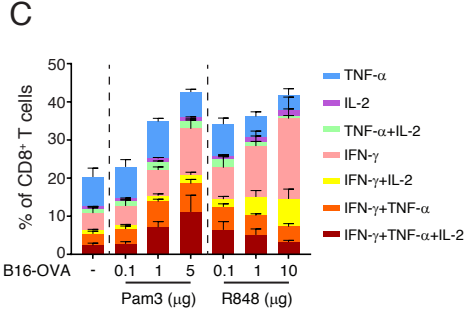
Salerno, Freen-van Heeren *et al.* Figure 3



Salerno, Freen-van Heeren *et al.* Figure 4



Salerno, Freen-van Heeren *et al.* Figure 5



Salerno, Freen-van Heeren *et al.* Figure 6